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(54) Title: LIGANDS TO ENHANCE CELLULAR UPTAKE OF BIOMOLECULES

(57) Abstract

Oligodeoxynucleoside methylphosphonate neoglycopeptide conjugates and related compounds for tissue specific delivery of biologically stable, non-ionic oligodeoxynucleoside analogs into cells.

LIGANDS TO ENHANCE CELLULAR UPTAKE OF BIOMOLECULES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a delivery system for introducing homogeneous oligonucleoside conjugates that are resistant to biodegradation into cells in a tissue specific manner via ligand directed, receptor mediated, endocytosis pathway.

2. Background Information

The antisense (anticode) or antigene strategy for drug design is based on the sequence-specific inhibition of protein synthesis due to the binding and masking of the target mRNA or genomic DNA, respectively, by the synthetic oligodeoxynucleotides (oligo dN) and their analogs (1). Implicit in this strategy is the ability of oligo-dNs to cross cellular membranes, thereby gaining access to the cellular compartments containing their intended targets, and to do so in sufficient amounts for binding to those targets to take place. Among the many oligo-dN analogs for application as antisense, non-ionic oligonucleoside methylphosphonates (oligo-MPs) have been extensively studied (*2*). Oligo-MPs are totally resistant to nuclease degradation (*3*) and are effective antisense agents with demonstrative *in vitro* activity against herpes simplex virus type 1 (*4*), vesicular stomatitis virus (*5*) and human immunodeficiency virus (*6*), and are able to inhibit the expression of *ras* p21 (*7*). For oligo-MPs to exhibit antisense activity, however, they must be present in the extracellular medium in

N-acetylgalactosamine neoglycopeptide, YEE(ah-GalNAc)$_3$ (*15*), conjugated to human serum albumin which was in turn linked to poly-L-lysine was shown to effectively deliver DNA into Hep G2 cells (*16*). While improved, these methods of delivery have several disadvantages: (1) by virtue of the structural heterogeneity of the starting materials (e.g. most often poly-L-lysine or bovine serum albumin) and the synthetic strategies employed, glycoconjugates derived from these materials are functionally equivalent, but structurally heterogeneous, therefore, their physical and biological properties would be difficult to fully define; (2) polycationic compounds (e.g. poly-L-lysine and cationic lipids) are toxic at concentrations employed for the delivery of DNA and oligo-dNs *in vitro* and presumably *in vivo*; (3) the ratio of oligo-dN or DNA to cationic conjugate must be empirically determined in each case.

A number of synthesis products have been described for the delivery of oligo dN which are heterogeneous mixtures of conjugates. Bonfils et al., for example, describe formation of conjugates between 6-phosphomannosylated protein and oligonucleosides which, because the modification of the protein and the formation of the disulfide link are not regiochemically controlled, yields a mixture of functionally related but structurally different molecules.

Several studies have described intracellular delivery of oligodeoxynucleotides or DNA which contain biodegradable phosphodiester internucleotide linkages. Because of this, they may have relatively short half lives within the cell and efficacy is consequently reduced. For example, an all phosphodiester 16-mer, d(T)$_{16}$, was extensively

SUMMARY OF THE INVENTION

It is an object of the invention to provide homogeneous oligodeoxynucleoside methylphosphonate conjugates, which contain non-biodegradable methylphosphonate internucleotide linkages. This enables the delivery of biologically stable, non-ionic oligodeoxynucleoside analogs into cells.

It is a further object of the invention to provide a method for synthesizing conjugates of oligodeoxynucleoside chimeras that contain all 2′-O-methylribose nucleosides and internucleotide linkages that alternate between methylphosphonate and phosphodiester or any other biostable oligomers. Such biostable oligomers include, but are not limited to, oligodeoxynucleotide analogs that contain: all 2′-deoxyribose nucleosides and internucleotide linkages that alternate between phosphorothioate and methylphosphonate; all 2′-deoxyribose nucleosides and phosphorothioate internucleotide linkages; all 2′-O-methylribose and phosphorothioate internucleotide linkages.

It is a further object of the invention to provide biologically non-degradable (or hydrolytic enzyme resistant) conjugates comprising oligo dN and/or oligo dN analogs which can efficiently cross cellular membranes and gain access to the cytoplasm. The term "efficiently", as used herein, is intended to mean that, for example, if the conjugate is present in the extracellular medium, then following a 24 hour incubation period at 37°C, the intracellular concentration will be at least approximately 3 times and preferably approximately 10 times the extracellular concentration.

It is a further object of the invention to provide a structurally defined and chemically uniform

the various chemical environments encountered in the extra- and intracellular medium.

The ligands for this delivery system include, but are not restricted to those shown in Fig. 1. The term "attachment groups", as used herein, refers to these ligands. The ligands consist of a synthetic, chemically defined, structurally homogeneous oligopeptide scaffold that is glycosylated with any of a number of sugar residues including, but not restricted to: glucose; N-acetylglucosamine; galactose; N-acetylgalactosamine; mannose; and fucose. The term "neoglycopeptide", as used herein, refers to these and similar structures. In addition, these oligopeptides provide frameworks to construct multivalent ligands with folic acid.

The term "pro-drug", as used herein, means a compound that, upon hydrolysis or bioreduction of specific chemical linkage(s), is released from the conjugate to become active or more active than when contained as part of the conjugate.

The term "chemically uniform", as used herein, means that at least 95% of the delivery assembly, and most preferably 99%, is a single species both in composition and in connectivity. Determination of chemical uniformity is by polyacrylamide gel electrophoresis, reverse-phase high pressure liquid chromatography, nuclear magnetic resonance, mass spectrometry and chemical analysis. The phrase "chemically defined and structurally homogeneous" is used interchangeably with "chemically uniform".

The term "gene specific", as used herein, means that the pro-drug is an oligonucleoside (particularly an oligodeoxynucleoside methylphosphonate or analog thereof) having a sequence that is complementary to a portion of a gene or a portion of a mRNA molecule

experimental section. Each data point represents the average of three trials ± one standard deviation.

Figure 6. 24 hour time course for the uptake of conjugate **10** by Hep G2 cells. Cells were incubated at 37°C and the cells collected as described in the experimental section. Each data point represents the average of three experiments ± one standard deviation.

Figure 7. Tissue specific uptake of conjugate **10** by Hep G2, HL-60 and HT 1080 cells. Cells were collected and the amount of [^{32}P] determined at 3 and 24 h for each cell line. Experiments were done in triplicate and the data expressed as the average ± one standard deviation.

Figure 8. Tissue Distribution of conjugate 10 and conjugate 12, which was produced by removing the terminal GalNAc residues of conjugate 10 with N acetylglucosamidase. Panel A: Percent initial dose per gram tissue versus time post-injection for conjugate 10. Panel B: Percent initial dose per gram tissue versus time post-injection for conjugate 12.

Figure 9. Structure of the Tracer, 3' conjugate.

Figure 10. Reaction scheme for the automated synthesis with 5'-thiol modifier.

Figure 11. Reaction scheme for the synthesis of 1c.

Figure 12A: Structure of **10**. The conjugate was synthesized with radioactive phosphate located on the

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

For convenience, the following abbreviations are used: AET, 2-aminomercaptoethanol (aminoethanethiol); ATP, adenosine triphosphate; BAP, bacterial alkaline phosphatase; CPG, controlled pore glass support; DIPEA, diispropylethylamine; D-MEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate buffered saline; DTT, dithiothreitol; EDAC, 1-ethyl-3-[3(dimethylamino)propyl] carbodiimide; EDTA, ethylenediaminetetraacetate; FCS, fetal calf serum; GalNAc, N-acetylgalactosamine; MEM, minimal essential medium with Earle's salts; SMCC, N-hydroxysuccinimidyl 4 (N-methylmaleimido)cyclohexyl-1 carboxylate; Tris, tris(hydroxymethyl)amine.

Synthesis of [5'-^{32}p]-[YEE(ah-GalNAc)$_3$]-SMCC-AET-pU^{m}p$\underline{T}_7$ (10). Materials. Methylphosphonamidite synthons were a generous gift from JBL Scientific, Inc., and are commercially available. They can readily synthesized from the nucleoside according to established procedures by an ordinarily skilled practitioner. All other reagents for the automated synthesis of **U^{m}pT$_7$** were purchased from Glen Research, Inc. HiTrap Q anion exchange columns were purchased from Pharmacia LKB Biotechnology. Reverse phase high performance liquid chromatography was carried out using Microsorb C-18 column purchased from Rainin Instrument Co., Inc. Cystamine hydrochloride, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC), 1-methylimidazole, and anhyd. dimethylsulfoxide (DMSO), dithiothreitol (DTT), and

group with bacteriophage T4 polynucleotide kinase and ensured reasonable stability of the phosphodiester due to the presence of the 2′-O-methyl group. The crude 8-mer was purified by HiTrap Q anion exchange chromatography (load with buffer containing <25% acetonitrile; elute with 0.1 M sodium phosphate, pH 5.8) and preparative reverse phase chromatography (Microsorb C-18) using a linear gradient (Solvent A: 50 mM sodium phosphate, pH 5.8, 2% acetonitrile; Solvent B: 50 mM sodium phosphate, pH 5.8, 50% acetonitrile; gradient: 0-60% B in 30 min). The oligomer thus purified was ca 97% pure by analytical HPLC, only contaminated by a small amount of the n-1 species.

Example 2
Synthesis of
[5′-^{32}P]-5′-O-[(N-2-thioethyl)phosphoramidate]-U^{m}p<u>T</u>, (9). The purified oligomer (168 nmol), ATP (160 nmol), H_2O (75 μL), 10x PNK buffer (5 mM DTT, 50 mM Tris•HCl, 5 mM $MgCl_2$, pH 7.6; 10 μL), [γ-^{32}P]-ATP (3000 Ci/mmol, 100 μCi, 10 μL), and PNK (150 U in 5 μL) were combined and incubated at 37°C for 16 h and evaporated to dryness. The residue was redissolved in 0.2 M 1-methylimidazole, pH 7.0 (100 μL) and 1.0 M cystamine hydrochloride, pH 7.2, containing 0.3 M EDAC (100 μL) and heated at 50°C for 2 h (*18*). The excess reagents were removed by SepPak (loaded with 50 mM sodium phosphate, pH 5.8, 5% acetonitrile; washed with 5% acetonitrile in water; eluted with 50% acetonitrile in water). The solvent was evaporated *in vacuo* and crude cystamine adduct redissolved in 10 mM phosphate containing 50 mM DTT (200 mL) and heated to 37°C for 1 h. The buffer salts and the excess reductant were removed from the reaction mixture as

contained in the conjugate.) Pneumatically assisted electrospray mass spectrometry produced a parent ion (negative ion mode) at M/Z 4080 (calculated mass 4080.7).

Cellular Uptake Experiments.

Materials. Minimal essential medium with Earle's salts supplemented with L-glutamine (MEM), Dulbecco's modified Eagle's medium (D-MEM), RPMI medium 1640 supplemented with L-glutamine (RPMI), Dulbecco's phosphate buffered saline (D-PBS), fetal calf serum (FCS), sodium pyruvate (100 mM), non-essential amino acids (10 mM), aqueous sodium bicarbonate (7.5%), and trypsin (0.25%; prepared in HBSS with 1.0 mM EDTA) were purchased from GIBCO BRL. Human hepatocellular carcinoma (Hep G2), human fibrosarcoma (HT 1080), and human promyleocytic leukemia (HL-60) cells were purchased from ATCC and were maintained in 1 x MEM supplemented with 10% FCS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Hep G2), 1 x D-MEM supplemented with 10% FCS (HT-1080), or 1 x RPMI supplemented with 10% FCS (HL-60). Silicon oil was a gift from General Electric (product no. SF 1250). Cells were counted using a Coulter Cell Counter.

Example 4

Uptake Experiments with Hep G2 Cells or HT 1080. Cells were passaged into 2 cm wells and grown in the appropriate medium to a density of ca. 10^5 cells per well. The maintenance media was aspirated and the cells were incubated at 37°C with 0.5 mL medium that contained 2% FCS and was made 1 μM in [5'-^{32}P]-labeled **10**. After the prescribed time had elapsed, a 5 μL aliquot of the media was saved for scintillation

synthesize a wide variety of useful conjugates. Fourteen examples of oligonucleoside analogs are shown in Table 1. Table 2 lists 14 examples of 3'- and 5'-phosphate modification, which provide a 1° amine or a thiol for further reaction. Table 3 shows the neoglycopeptide, which contains a N-terminal amino group, and four methods for modifying the amine to provide a thiol. Finally, Table 4 lists several heterobifunctional cross-linking reagents and a Cathepsin D sensitive oligopeptide, which can be used to link the pro-drug to the ligand. It will be readily apparent that many other reagents are available which would be suitable.

In general, there are two reaction schemes that may be employed to covalently join the oligomer and neoglycopeptide. The first entails the coupling of an oligomer and the neoglycopeptide using a heterobifunctional cross-linking reagent and can be classified as a three component reaction. This scheme provides for complete regiochemical control of the coupling reaction and yields structurally defined and homogeneous conjugates. For example, if an oligomer of the type shown in Table 1, entry 1, were modified at its 5'- end with a thiol linker (Table 2, entry 10) post-synthetically and conjugated to YEE(ah-GalNAc)$_3$ (Table 3, entry 1) with SMCC (Table 4, entry 3), a conjugate with a linkage identical to the following would be obtained:

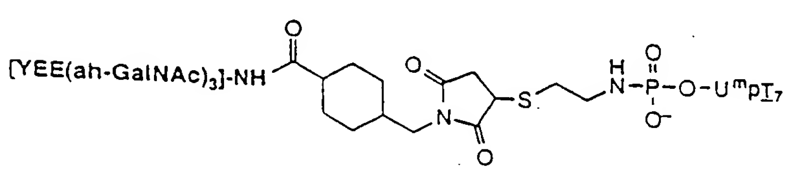
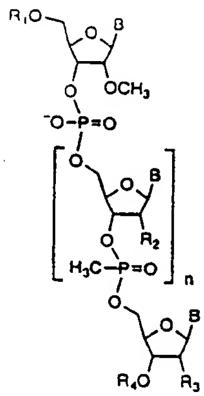


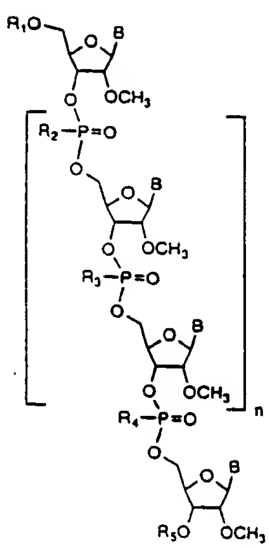
Table 1. Oligonucleoside methylphosphonate analogs.



Entry	R_1	R_2	R_3	R_4
1	5'-conjugate	H	H	H
2	H	H	H	3'-conjugate
3	5'-conjugate	$-OCH_3$	$-OCH_3$	H
4	H	$-OCH_3$	$-OCH_3$	3'-conjugate

B = A, C, G, or T

$8 \leq n \leq 50$

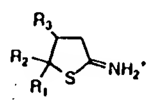
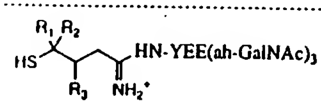
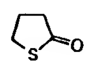
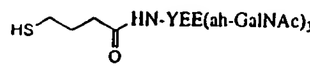
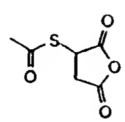
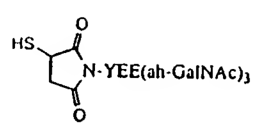
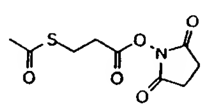
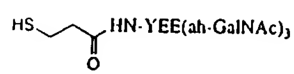


Entry	R_1	R_2	R_3	R_4	R_5
5	5'-conjugate	O^-	CH_3	O^-	H
6	H	O^-	CH_3	O^-	3'-conjugate
7	5'-conjugate	CH_3	O^-	CH_3	H
8	H	CH_3	O^-	CH_3	3'-conjugate
9	5'-conjugate	S^-	CH_3	S^-	H
10	H	S^-	CH_3	S^-	3'-conjugate
11	5'-conjugate	CH_3	S^-	CH_3	H
12	H	CH_3	S^-	CH_3	3'-conjugate
13	5'-conjugate	S^-	S^-	S^-	H
14	H	S^-	S^-	S^-	3'-conjugate

B = A, C, G, or T

$8 \leq n \leq 50$

Table 3. Illustrations of functional group modifications to YEE(ah-GalNAc)$_3$.[a]

Entry	Modifying Reagent	Ligand	Reactive Group	Reactivity
1	none	H_2N-YEE(ah-GalNAc)$_3$	amine	active esters isothiocyanates isocyanates aldehydes
2[b]			thiol	1° halides maleimides activated disulfides
3			thiol	
4			thiol	
5			thiol	

[a]These reagents may be used to modify any of the ligands illustrated in Figure 1.
[b]See Goff, D. A.; Carroll, S. F. (1990) Substituted 2-iminothiolanes: reagents for the preparation of disulfide cross-linked conjugates with increased stability *Bioconjugate Chem. 1*, 381-386.

Discussion

Synthesis of [YEE(ah-GalNAc)$_3$]-SMCC-AET-pU^{m}p$\underline{T}_7$ (10). Synthesis and purification of YEE(ah-GalNAc)$_3$ (5) (*15a*) and U^{m}p$\underline{T}^7$ (6) (*17*) was carried according to established procedures. In order to form a covalent link between **5** and **6**, the 5' end of **6** was modified using the method of Orgel (*18*). This introduced a disulfide into the oligo-MP, which in turn could be reduced with DTT to give a 5'-thiol. The neoglycopeptide **5** was modified in a complementary fashion using the heterobifunctional cross-linking reagent, SMCC, capable of combining with the *N*-terminal amino group of **5**. Coupling of the maleimido group introduced by SMCC and the 5'-thiol of the modified oligo-MP resulted in linkage of the oligo-MP and neoglycopeptide via a metabolically stable thioether (Figure 2). To begin the synthesis, **6** was phosphorylated using T4 polynucleotide kinase and 0.95 equivalents of ATP. Formulation of the end-labeling reaction in this way ensured that ca. 90% of the ATP was consumed allowing efficient use of the [^{32}P]-ATP to radioactively label the conjugate. Modification of the 5' phosphate was accomplished in two steps. The end-labeled oligo-MP was incubated at 50°C with 0.5 M cystamine hydrochloride in a buffer containing 0.1 M 1-methylimidazole at pH 7.2 in the presence of 0.15 M EDAC to give the 5' cystamine phosphoramidate in 65% yield. Up to 35% of thymidine-modified oligo-MP was produced during this reaction and, despite attempts to modify the reaction conditions (e.g., lowering the temperature and reducing the concentration of EDAC), its production could not be eliminated. This side product presumably arises due to reaction of EDAC with N-3 of

2 h followed by overnight incubation with an aqueous solution 0.3 M ethylenediamine hydrochloride buffered to pH 7.0. (Miller, P.S.; Levis, J. T., unpublished results). This modification prevents removal of the 5'-phosphate by cellular phosphatase activity.

In each instance, the modified oligo-MP was present at a concentration of 1 μM in medium containing 2% fetal calf serum (FCS) and incubations were carried out at 37°C. The conjugate rapidly associated with the cells when incubated alone, loading the cells in a linear fashion to the extent of 7.8 pmol per 10^6 cells after only two hours (Figure 5). In contrast, when a 100-fold excess of free **5** was present with 1 μM conjugate, association of **10** was only 0.42 pmol per 10^6 cells, a value essentially identical to that obtained with the control oligo-MP **11** (0.49 pmol per 10^6 cells). As an additional control, Hep G2 cells were incubated with **11** in the presence of a 10-fold excess of **5** to assess the possibility that despite the absence of a covalent link between **5** and **11**, **5** could cause uptake of **11** by the Hep G2 cells. The amount of cell associated **11** following a two-hour incubation was only 0.60 pmol per 10^6 cells, significantly less than found with the conjugate **10**. In addition, the uptake of **10** by Hep G2 cells for longer times was examined (1 μM conjugate, 37°C), and found to be linear up to ca. 24 hours reaching a value of 26.6 pmol per 10^6 cells (Figure 6). The results of these experiments indicate that: (1) the conjugate **10** associates with Hep G2 cells by binding specifically to the asialoglycoprotein receptor; (2) a covalent link between the oligo-MP and neoglycopeptide is essential for significant enhancement of the association of the oligo-MP with Hep G2 cells; and (3) uptake of **10** by

capable of delivering the oligo-MP 6 in a highly selective manner to hepatocytes.

Whole animal biodistribution and pharmacokinetics

Synthesis of [YEE(ah-GalNAc)$_3$]-SMCC-AET-[^{32}P]-p$U^{m}p\underline{T}_7$ (10) and [YEE(ah)$_3$]-SMCC-AET-[^{32}P]-p$U^{m}p\underline{T}_7$ (12). Briefly, the parent oligodeoxynucleoside methylphosphonate (oligo-MP), $U^{m}p\underline{T}_7$, was 5′ end-labeled with [γ-^{32}P]-ATP and ATP to give p*$U^{m}p\underline{T}_7$ having a specific activity of 300 μCi/14 nmol (the * indicates the position of the radioactive nuclide). The 5′ phosphate was modified with cystamine in the presence of 1-methylimidazole and water soluble carbodiimide. The resulting disulfide was reduced with excess dithiothreitol and conjugated with the ligand, YEE(ah-GalNAc)$_3$, using the heterobifunctional cross-linking reagent SMCC. The conjugate (1) was purified by polyacrylamide gel electrophoresis, extracted from the gel and desalted using a SepPak cartridge. The pure conjugate was characterized both enzymatically and chemically. A portion of the conjugate was treated with N-acetylglucosamidase in order to completely remove the GalNAc residues (**12**). Both **10** and **12** were >99% pure as judged by PAGE analysis. The solutions containing the conjugates were placed in sterile test tubes and lyophilized under aseptic conditions in preparation for the whole animal biodistribution and pharmacokinetic experiments.

Whole animal biodistribution and pharmacokinetics. The conjugates **10** and **12** were redissolved in sterile water. Each mouse received 0.11 μCi (7 pmol) of conjugate **10** and 0.036 μCi (1.2

the percent of initial dose per gram of tissue was 40%, 5.0% and 1.1% for the bladder/urine, kidney and liver, respectively.

It is apparent from these data that: (1) conjugate **10** associates specifically with the liver; (2) association of conjugate **10** is wholly dependent upon the presence of the GalNAc residues on the ligand; (3) conjugate **10** or, more likely, its metabolites, are cleared from the liver within 24 hours and eliminated from the mouse via the kidney and, hence, finds its way into the bladder and urine. Furthermore, owing to the low level of radioactivity found in the blood versus the large amount of radioactivity associated with the liver, it can be concluded that conjugate **10** is delivered into the hepatocytes rather than simply associated with the liver within the interstitial space.

Synthetic Procedures for Compounds 1b and 1c

Additional examples of compounds of the invention are shown in Table 5.

Sequences 1-3 can be linked with substituent groups indicated as oligonucleotides a-e at the bottom of Table 5 using the synthesis methods described hereinbelow to form further examples of compounds of the invention. For example, 1b consists of sequence 1 with substituents according to the invention of C6-thiol-ps, O^-, CH_3, and 3'-conjugate (the structure of which is shown in Fig. 9). Compounds of the structures indicated by 1b (compound 13) and 1c (compound 14) were synthesized according to the scheme shown in Figure 3, as set forth in detail in Examples 8 and 9. It will be clear that with suitable substitution in starting material and changes in the synthesis the other combinations can be similarly synthesized.

Example 7

Synthesis of SMCC-YEE(ah-GalNAc)$_3$ (8) (Alternative method). About 1-2 μmole of YEE(ah-GalNAc)$_3$ was dried into a 1 mL glass Reacti-vial. To this solution, anhydrous DMSO (250 μL) and anhydrous DIPEA (3 μL) was added, then treated with 150 μL of a solution containing vacuum-dried SMCC (6 mg) in anhydrous DMSO. The mixture was vortexed briefly and left stand at room temperature for 2 hours. Analysis by reversed-phase hplc indicated complete conversion of the starting YEE(ah-GalNAc)3 (elution time: 7.3 min) to the desired product SMCC-YEE(ah-GalNAc)$_3$ (elution time: 9.8 min). The reaction mixture was then diluted to 10 mL with 50 mM sodium phosphate (pH 5.8) containing 2% CH_3CN and was loaded onto a Sep-Pak cartridge. The cartridge was washed with 10 mL of 50 mM sodium phosphate (pH 5.8) containing 2% CH_3CN and the product was eluted with 10 mL of 25% CH_3CN/H_2O. The product

coupling step of the solid-phase synthesis. When necessary, the Beaucage reagent (Glen Research) was substituted for the low moisture oxidizer to effect sulfurization of the phosphite to give the phosphorothioate according to standard established procedures. The oligomers were deprotected under Genta one-pot method and were purified by trityl-on procedures. Final purification were conducted using a preparative reversed-phase C18 column.

The reduction of the disulfide moiety to the thiol was effected by the treatment of the 5'-disulfide-containing oligomers with DTT. Thus, a 2.5 OD_{260} (~16 nmole) disulfide oligomer was dissolved in 400 μL of freshly prepared and degassed 50 mM DTT solution in 10 mM sodium phosphate, pH 8. The mixture was incubated at 37°C for 2 hr. Quantitative reduction were confirmed by reversed-phase HPLC analysis, which shows that the thiol oligomers elute faster than the parent disulfide oligomers. The thiol oligomer was then purified on a Sephadex G-25 column (10x300mm) to remove DTT and salts. Column packing and sample elution were effected by the use of degassed 20% ethanol-water. The G-25 fraction containing the pure thiol oligomer was used immediately in the next reaction to minimize unwanted oxidation.

Example 9

Synthesis of SMCC-YEE(ah-GalNAc)$_3$-containing Oligonucleotides (compound 14, shown as 1c in Table 5). The G-25 fraction containing 1.8 OD_{260} (12 nmole) pure thiol oligomer (1b) was mixed with SMCC-YEE(ah-GalNAc)$_3$ (50 nmole) immediately after it was collected. The mixture was concentrated to dryness in a speed-vac. The residue was dissolved in

was aspirated and the cells were incubated at 37°C with 0.5 ml DMEM containing 2% FCS and made 1uM in [5'^{32}p] with 1c, 1d, or 1e. All other methods were identical to those followed in Example 4.

In order to measure the efflux of 1c, HepG2 2.2.15 cells were seeded and incubated with 1 uM of the conjugated oligomer for twenty-four hours as described above. The oligomer containing medium was then aspirated and the cells washed twice and subsequently incubated in 0.5 ml maintenance medium. At designated times the cells were collected and lysed as described in the Cellular Uptake section of the Provisional Application. Efflux was determined by monitoring the amount of radioactivity and by inference the concentration of the conjugated oligomer in the cell lysate.

DISCUSSION

The cellular uptake experiments described hereinabove were extended to examine the cellular association of 1d with Hep 2G 2.2.15 cells. As in the case of the oligo-mp, a control was prepared by modifying the ^{32}P labeled 5' end of 1 with ethylenediamine to yield 1e.

The results of this experiment were very similar to those performed with the modified oligo-mp. The conjugated 2'OMe alternating oligomer (1d) was taken up by Hep2G 2.2.15 cells in a linear fashion to the extent of 7.7 pmoles/10^6 cells after two hours incubation (Table 6).Uptake increased to 14.2 pmoles/10^6 cells in three hours and peaked at 28.5 pmoles/106 cells after twenty-four hours incubation. In contrast, the EDA modified oligomer (1e) associated with Hep G2 2.2.15 cells to the extent of 0.275 pmoles/10^6 cells after two hours,.978 pmoles/10^6

TABLE 6-Uptake of conjugated YEE(ah-GAlNac)$_3$-SMCC-AET-2'0-Me $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$ (1d) and EDA-2'-0-Me- $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$ (1e) by Hep 2G 2.2.15 cells in culture (pmoles/10^6 cells)

OLIGOMER	1 HOUR	2 HOURS	3 HOURS	24 HOURS
1d	3.63	7.71	14.16	28.52
1e	0.277	0.305	0.400	0.450

TABLE 7-Uptake of YEE(ah-GAlNac)$_3$-SMCC-S(CH$_2$)$_6$-*ps*- 2'0-Me- $^{5'}AG_pUC_pAG_pUC_pAG_pUC_pAG_pU^{3'}$ -U^{M}dT*$^{3'-3'}$ (dT-T)-^{32}P-EDA (1c) by Hep G2 2.2.15 cells in culture(pmoles/10^6 cells)

OLIGOMER	4 HOURS	8 HOURS	12 HOURS	16 HOURS	24 HOURS
1c	9.44	18.60	22.05	24.92	28.97

Example 11

Whole animal experiments were performed to test for the ability of a delivery vehicle of the invention, i.e, which contains the asialoglycoprotein ligand, YEE(ah-GalNAc)$_3$ radiolabeled with ^{32}P, to deliver synthetic oligo-MPs specifically to the liver of mice and to examine the metabolic fate of this conjugate in isolated Hep G2 cells and in vivo in mouse liver and urine.

For comparison, a conjugate which lacks the three terminal Gal NAc residues, was also synthesized. This sugarless conjugate served as a control for the study of ligand (GalNAc)-specific uptake in mice.

after sacrifice, the bladders were removed and placed into glass vials. Solvables® (NEN; 1 mL) was added to each sample. The samples were then placed on a slide warmer to be digested overnight and removed the next morning to cool. The digested samples were decolorized with 3 to 7 drops of H_2O_2 (30% w/v), and 10 mL Formula 989 (NEN) scintillation cocktail were added. The amount of radioactivity was determined by scintillation counting (Packard 1900 TR; <3% error). Aliquots of the injected dose were counted along with the samples to calculate the percent dose per organ or gram tissue.

Example 13

Analysis of metabolites isolated from Hep G2 Cells. Cells (ca. 10^5) were incubated in media containing 1 μM [^{32}P]-labeled 1 for 2, 4, 8, 16 and 24 h, washed with PBS (2x), pelleted through silicon oil and lysed (0.5% NP 40, 100 mM sodium chloride, 14 mM Tris-HCl pH 7.5, 30% ACN). The lysate was extracted with 50% aqueous acetonitrile (v/v) twice. The extracts were lyophilized, redissolved in formamide loading buffer and analyzed by PAGE (15%, 2 V/cm, 30 min).

Example 14

Analysis of Conjugate Metabolism. Male CD-1 mice, weighing between 22 to 35 g, received a single injection via tail vein of 40 pmole of [^{32}P]-[YEE(ah-GalNAc)$_3$]SMCC-AET-pU^{m}p$\underline{T}_7$ (10). Animals were sacrificed after 15, 60 and 120 minutes. Livers and bladders were collected as before, placed into plastic vials and immediately frozen (80°C). Samples of liver were thawed to 0°C and weighed (average mass 0.25 g). The tissue was homogenized (Polytron

In order to investigate the *in vivo* tissue and organ distribution of conjugate 10, mice were injected via tail vein with radiolabeled conjugate as described above and the amount of radioactivity associated with each organ determined by scintillation counting. Table 9 shows the conjugate associates to the greatest extent with the liver, reaching a value of 69.9% of the injected dose 15 minutes post-injection. The ranking of total radioactivity in the other tissues measured at 15 minutes post-injection was, in decreasing order: muscle > kidney > blood > spleen. The peak value of radioactivity for the urine was 28% of the injected dose and was reached after 30 minutes. The amount of radioactivity associated with the kidneys and blood decreased over time. It is noteworthy that, while it may be expected that metabolites of the conjugate produced in the liver would become deposited in the gastrointestinal tract via bile excretion, little radioactivity was associated with the gall bladder, upper and lower gastrointestinal tract, and feces (Table 9).

Table 11 shows that conjugate 12, which lacks the three terminal GalNAc residues, was distributed in the order: muscle > blood > kidneys > liver > spleen. The amount of muscle and liver radioactivity appeared to remain constant whereas that associated with the blood and kidneys decreased over the 24 hour study. The peak value of radioactivity in the urine was 39.9% at 30 minutes post-injection. As a control, an identical experiment was carried out with conjugate 10 (Table 11). The ranking of tissue distribution was, in order of decreasing amounts of radioactivity: liver >> muscle > kidney > blood >

intensity of this band occurs at 8 h followed by a gradual decrease to 24 hour. As was observed with Class I metabolites, all Class II metabolites appear to decrease in amount by the 24 hour time point. Class III metabolite(s) are largely immobile in the gel matrix and are, for the most part, retained in the well of the Polyacrylamide gel. The intensity of this band increases over time, reaching a maximal value at 24 hours.

Analysis of the metabolic fate of 10 in intact mouse liver was carried out in a similar fashion. Figure 14 shows the outcome of PAGE analysis of liver homogenate extracts obtained from liver samples of mice injected with [^{32}P]-labeled conjugate 10.

Following 15 minutes post-injection, there remains a significant amount of intact conjugate 10 (Class I metabolites, cf. Fig. 13). The resolution of the gel is not sufficient to permit discrimination between the two species. The remainder of the radiolabeled species in this sample migrated significantly faster than 1 and 2 and did not co-migrate with any of the controls. These metabolites appear to have a broader range of mobilities and the slowest are significantly less mobile than the Class II metabolites identified with Hep G2 cells (Class II'). At the later time points, nearly all intact 10 and 12 has disappeared, whereas the Class II' metabolites appear to increase in amount.

Figure 15 shows the pattern of metabolites observed in mouse urine following i.v. administration of the radiolabeled conjugate 10. Metabolites of Class I are the only radiolabeled species detected. The conjugate appears to be largely intact with a small but significant amount of material converted to

Discussion

The evidence described herein demonstrates that [^{32}P]-labeled conjugate 10, which is chemically defined and homogeneous, is capable of crossing the cellular membrane of Hep G2 cells in a manner that is both ligand and cell-type specific. A logical extension of these investigations was to determine the tissue distribution of 1 *in vivo* and to compare the metabolic fate of 10 *in vitro* and *in vivo* and to compare the data with those obtained with conjugate 12 which lacks the three terminal GalNAc residues.

The *in vivo* tissue distribution data confirm the results obtained with cultured human cells. Highly selective targeting of the oligodeoxynucleoside methylphosphonate to the liver (70±10% of i.d.) was effectively achieved through covalent attachment of the oligomer and the asialoglycoprotein receptor (ASGP) ligand, YEE(ah-GalNAc)$_3$. Indeed, the concentration of conjugate in the liver was 25-fold greater than that found in the blood and approximately 10-fold greater than in muscle based on whole tissue measurements (Table 9). These results compare favorably, and are in some ways superior, to the outcome of similar experiments reported by Lu et al., where the delivery of an [^{32}P]-labeled antisense oligo-dN to rat liver was enhanced when compared to other tissues owing to its complexation with an asialoglycoprotein-poly-L-lysine conjugate (Lu et al., 1994). As noted by the authors, however, the preference of the complex for the liver was marginal since the spleen, lungs and kidneys accumulated the radiolabeled oligo-dN as well (e.g., distribution for each tissue was ca. 6, 4, 2 and 2% of injected dose per gram, respectively, after 5 minutes post injection; Lu et al., 1994). It is of further

from the plasma into the kidney and urine. The HPLC study showed that the intact 12-mer was metabolized to 11-mer via enzymatic cleavage of the terminal nucleotide and both were eliminated rapidly into the urine after i.v. injection. Thus, the results reported herein agree well with the results obtained earlier, demonstrating the importance of the GalNAc terminal in directing the uptake of oligomer conjugate into liver.

In order to gain insight into the *in vitro* and *in vivo* metabolic fate of conjugate 10, we examined extracts obtained from Hep G2 cells grown in culture and from the liver and urine of mice by PAGE analysis. We noted that three classes of metabolites (Class I-III) were produced in Hep G2 cells and in mouse liver whereas only Class I metabolites were isolated from mouse urine. Class I metabolites appeared to arise owing to degradation of the ligand. Two enzymatic reactions were employed in an attempt to model the production of these species: N-acetylglucosamidase and chymotrypsin. The former treatment yielded 2, which migrated slightly faster than 1 due to the slight reduction in mass resulting from the loss of the three terminal GalNAc residues. The latter treatment resulted in a substantially enhanced mobility resulting from both the loss of a majority of the ligand and an increase in the overall charge from -1 to -2 (Fig. 12). These two model reactions produced compounds with modified ligands remaining covalently linked to intact radiolabeled oligo-MP. It is reasonable to conclude, therefore, that other species migrating to the same region of the gel resulted from degradation of the ligand and not from bond cleavage at other labile sites of 1. For example, hydrolysis of a single aminohexyl side

endosomal compartment resulted in the hydrolysis of the P-N bond, 1 was incubated at 37°C in 50 mM sodium citrate at pH 5.5 and 6.0. We observed that 1 was stable at pH 6 but was substantially hydrolysed to 4 at pH 5.5 (>50%) following 24 hours and that hydrolysis occurred specifically at the phosphoramidate P-N bond as determined by PAGE analysis (data not shown). Thus, it is reasonable to conclude that incorporation of radioactive phosphate into cellular structures occurs by hydrolysis of the P-N bond due to acidification of the endosomal compartment containing 1 and release of the terminal phosphate into the cellular milleau by phosphatase activity.

The profile of metabolites observed in extracts from Hep G2 cells includes each class of metabolites. At early time points, the majority of the radioactivity is contained in Class I species, chiefly 1 and 2. At later time points, the distribution of metabolites shifts from Class I to Class II and III, where at the last time point sampled, a majority of radioactive phosphorous resides with Class III metabolites, indicating substantial hydrolysis of the P-N bond had occurred over the course of the experiment. It is readily apparent that 1 is significantly metabolized once taken into Hep G2 cells, suggesting that intracellular delivery of an antisense oligo-MP, or other agents, would be feasible by this method.

Due to the fact that only the phosphorus at the N-P bond is labeled with ^{32}P,it is not possible to measure the metabolic fate of the oligonucleotide analoge. Since extensive metabolism of the oligonucleotide would adversely affect the ability to specifically interact with intracellular

these results indicate that this method for the delivery of antisense agents, either methylphosphonates or other analogs, and other therapeutically useful agents will be very useful. Furthermore, these results demonstrate the potential for diagnostic imaging procedures that utilize the tissue specificity of the ligand coupled to the nucleic acid specificity of the antisense moiety, providing the means to measure regional abnormalities of cellular functions *in vivo* with heretofore unrealized specificity.

Table 10. Percent injected dose accumulated per organ following intravenous injection of [^{32}P]-[YEE(ah-GalNAc)$_3$)]-SMCC-AET-pU^{m}pT$_7$.

	percent injected does per organ time post injection (min.)				
Organ	15	30	60	120	1440
Blood[a]	1.71±0.32	1.55±0.23	0.87±0.12	1.00±0.37	0.44±0.13
Liver[a]	42.4±8.0	28.9±0.97	21.7±3.0	18.6±6.5	2.89±0.45
Spleen[a]	0.04±0.02	0.08±0.01	0.16±0.03	0.23±0.04	0.30±0.11
Kidneys[a]	0.93±0.35	1.17±0.11	1.18±0.06	1.15±0.13	0.68±0.13
Muscle[a]	9.95±1.04	8.37±1.26	8.85±1.30	8.62±0.97	8.63±1.16

[a]Values are reported as the average percent injected dose per organ three animals ± one standard deviation. Approximately 0.1 microCi (7 pmol) intravenously into each mouse. The following values were used to determine the percent dose per organ from percent dose per gram of tissue; mass of blood =0.07 x body mass; mass of liver = 1.14 g; mass of spleen = 0.124 g; mass of kidneys =0.4 g; mass of muscle=0.4 x body mass. The average body mass was 23.7+1.2 (std. dev.; n=15). The peak value of radioactivity in the urine was 17.1±10.2% of injected dose at 30 minutes. The large standard deviation reflects the variation in urine production and completeness of collection between individual animals.

It will be appreciated that a variety of useful compounds can be synthesized using the methods described herein, particularly with the reagents and compounds detailed in Tables 1-4. The current examples are not meant to be limiting but rather merely illustrative. It will be clear that various modifications can be made and these are intended to be included in the scope of the claimed invention.

References refered to herein are listed below for convenience and are hereby incorporated herein by reference:

1. Mirabelli, C K.; Crooke, S.T. (1993) Antisense oligonucleosides in the context of modern molecular drug discovery and development, in Antisenese research and applications (Crooke, S.T., and LeBleu, B. Ed.) CRC Press, Boca Raton, pp. 7-35.

2. Ts'o, P.O.P.; Aurelian, L.; Chang, E.; Miller, P.S. (1992) Nonionic oligodeoxynucleotide analogs (Matagen™) as anticodic agents in duplex and triplex formation. Ann. NY Acad. Sci. 600, 159-177.

3. Miller, P.S.; McParland, K.B.; Javaraman, K.; Ts'o, P.O.P. (1981) Biochemical and biological effects of nonionic nucleic acid methylphosphonates. Biochemistry 20, 1874-1880.

4. (a) Smith, C.C.; Aurelian, L.; Reddy, M.P.; Miller, P.S.; Ts'o, P.O.P. (1986) Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. Proc. Natl. Acad. Sci. USA 83, 2787-2791. (b) Kulka, M; Smith, C.C.; Aurelian, L.; Fishelevich, R.; Meade, K.; Miller, P.;

Virology 62, 3914-3917. (c) Laurence, J.; Sikder, S.K.; Kulkosky, J.; Miller, P.; Ts'o P.O.P. (1991) Induction of chronic human immunodeficiency virus infection is blocked by a methylphosphonate oligodeoxynucleoside targeted to a U3 enhancer element. J. Virology 65, 213-219.

7. (a) Brown, D.; Zhipeng, Y.; Miller, P.; Blake, K.; Wei, C.; Kung, H.-F.; Black, R.J.; Ts'o, P.O.P., Chang, E.H. (1989) Modulation fo ras expression by anti-sense, non-ionic deoxyoligonucleotide analogs. Oncogene Research 4, 243-252. (b) Yu, Z.; Chen, D.; Black, R.J.; Blake, K.; Ts'o, P.O.P.; Miller, P.; Chang, E.H. (1989) Sequence specific inhibition of *in vitro* translation of mutated or normal ras p21. J. Experimental Pathogy 4, 97-107. (c).Chang, C.H.; Miller, P.S.; Cushman, C.; Devadas, K.; Pirollo, K.F.; Ts'o, P.O.P.; Yu, Z.P. (1991) Antisense inhibition of ras p21 expression that is sensitive to a point mutation. Biochemistry 30, 8283-8286.

8. (a) Wu, G.Y.; Wu, C.H. (1987) Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. J. Biol. Chem. 262, 4429 4432. (b) Wu, G.Y.; Wu, C.H. (1988) Receptor-mediated gene delivery and expression *in vivo*. J. Biol. Chem. 263, 14621-14624. (c) Wu, G.Y.; Wu, C.H. (1988) Evidence for targeted gene delivery to Hep G2 hepatoma cells *in vitro*. Biochemistry 27, 887-892. (d) Wu, G.Y.; Wu, C.H. (1992) Specific inhibition of hepatitis B viral gene expression *in vitro* by targeted antisense oligonucleotides. J. Biol. Chem. 267, 146-12439.

9. Plank, C.; Zatloukal, K.; Cotten, M.; Mechtler, K.; Wagner, E. (1992) Gene transfer into hepatocytes

E.; Plank, C.; Zatloukal, K.; Cotten, M.; Birnstiel, M.L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. Biochemistry 89, 7934-7938.

14. Bonfils, E.; Dupierreux, C.; Midoux, P.; Thuong, N.T.; Monsigny, M.; Roche, A.C. (1992) Drug targeting: synthesis and endocytosis of oligonucleotide-neoglycoprotein conjugates. Nucleic Acids Res. 20, 46214629.

15. (a) Lee, R. T.; Lee, Y. C. (1987) Preparation of cluster glycosides and N-acetylgalactosamine that have sub-nanomolar binding constants toward mammalian hepatic Gal/GalNAc-specific receptors. Glycoconjugate J. 4, 317-328. (b) Oshumi, Y.; Ichikawa, Y.; Lee, Y. C. (1990) Neoglycoproteins: Recent Progress and Future Outlook. Cell Technology 9, 229-238.

16. Merwin, J.R.; Noell, G.S.; Thomas, W.C.; Chion, H.C.; De Rome, M.E.; McKee, T.D.; Spitalny, G.L.; Findeis, M.A. (1994) Targeted delivery of DNA using YEE(ah-GalNac)$_3$, a synthetic glycopeptide for the asialoglycoprotein receptor. Bioconjugate Chem. 5, 612-620.

17. (a) Miller, P.S.; Cushman, C.D.; Levis, J.T. (1991) Synthesis of oligo-2'-deoxyribonucleoside methylphosphonates, in Oligonucleotides and analogues. A practical approach (Eckstein, E., Ed.) IRL Press, Oxford, pp. 137-154. (b) Hogrefe, R. I.; Reynolds, M.A.; Vaghefi, M.M.; Yang, K.M.; Riley, K.M.; Klem, R.E.; Arnold, L.T., Jr. (1993) An

WHAT IS CLAIMED IS:

1. A chemically uniform conjugate of formula

A-L-P

wherein

A represents an attachment group,

P represents a biologically stable pro-drug that is released from the conjugate following hydrolysis or reduction of specific biochemical linkages, and thereby becomes active or more active than when contained as part of the conjugate, and

L represents a bifunctional linker that is chemically combined with the attachment group and pro-drug regiospecifically to provide a chemically uniform conjugate, and

A, L, and P are covalently linked.

2. A conjugate according to claim 1, wherein A binds specifically to cell surface receptors unique to a specific tissue, thereby providing a means for tissue- or cell- specific targeting of the pro-drug.

3. A conjugate according to claim 1 wherein A is a ligand which binds specifically to a cell surface receptor and thereby facilitates the entrance of the conjugate into cells having said receptor.

4. A conjugate according to claim 1 wherein A is a neoglycopeptide.

5. A conjugate according to claim 1 wherein A is selected from the compounds of Figure 1.

6. A conjugate according to claim 1 wherein P is an oligonucleoside with internucleotide linkages resistant to enzymatic hydrolysis or biodegradation.

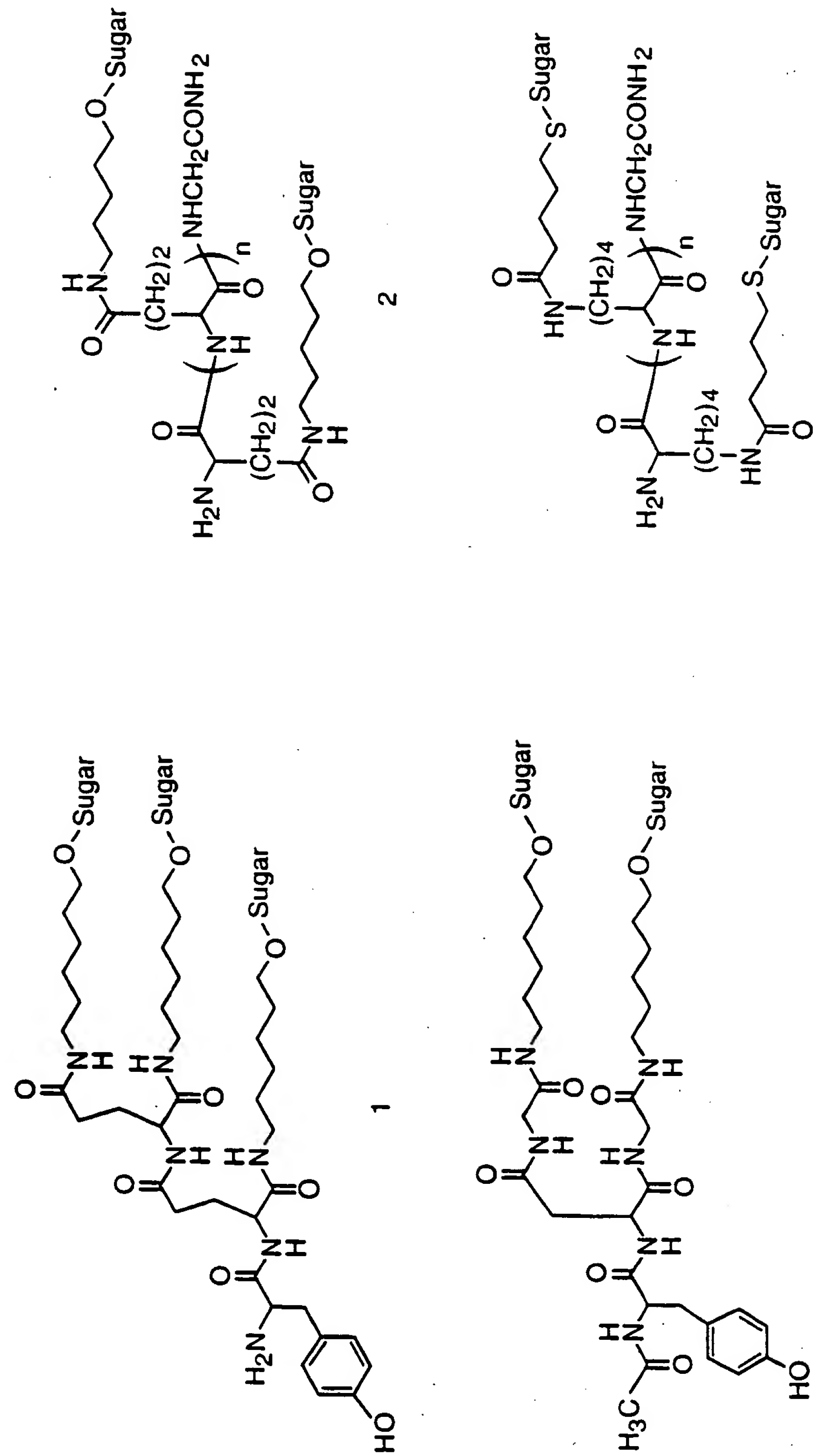
oligodeoxynucleoside methylphosphonate or an analog thereof, and said oligomer and said neoglycopeptide are covalently linked.

15. The delivery system of claim 14 which is gene specific.

16. The delivery system of claim 15 which is sequence specific.

17. The delivery system of claim 16 wherein the tissue is liver.

FIG. 1



1

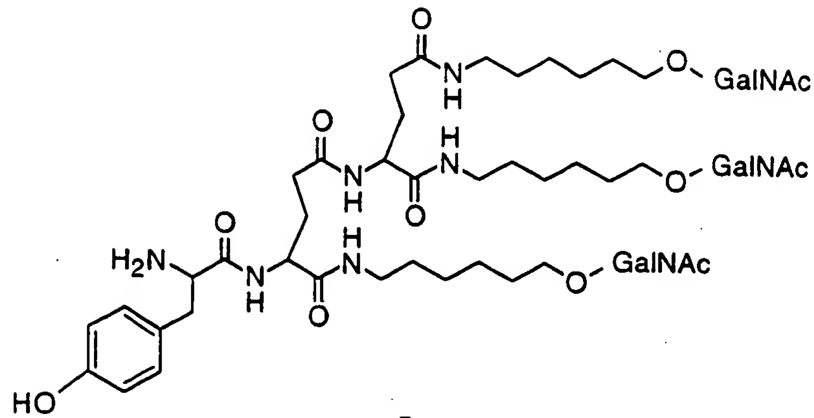
2

3

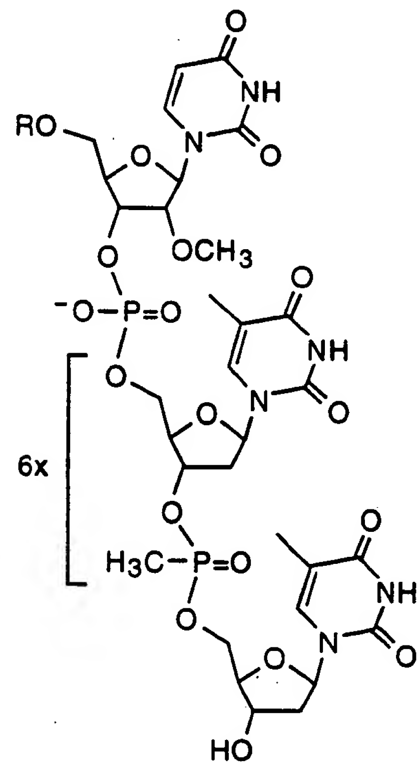
4

a Sugar may be, but is not restricted to, any of the following sugars: glucose, N-acetylglucosamine, galactose, N-acetylgalactose, mannose, fucose.
b Folic acid may be used in place of the sugar residues.

FIG. 2



5



6 R = H

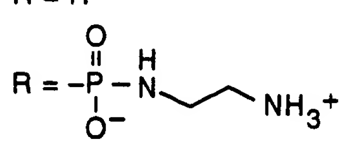
11 R = 

FIG. 3

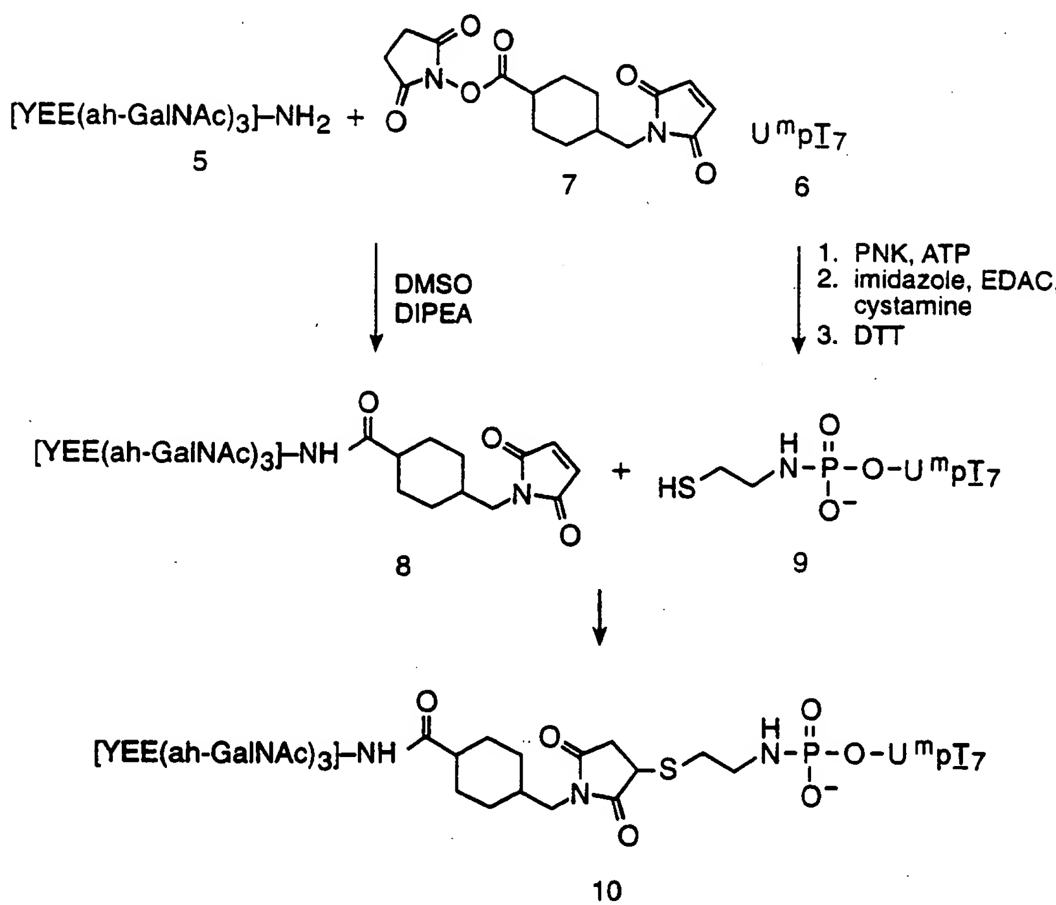

[YEE(ah-GalNAc)3]-NH2
5
7
UmpI7
6
DMSO
DIPEA
1. PNK, ATP
2. imidazole, EDAC, cystamine
3. DTT
[YEE(ah-GalNAc)3]-NH
8
9
[YEE(ah-GalNAc)3]-NH
10

FIG. 4

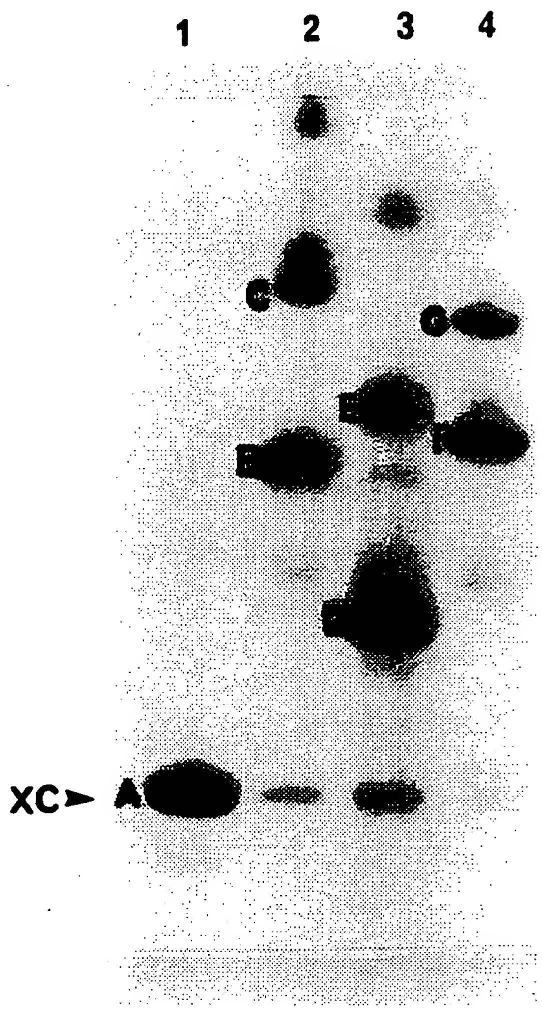


FIG. 5

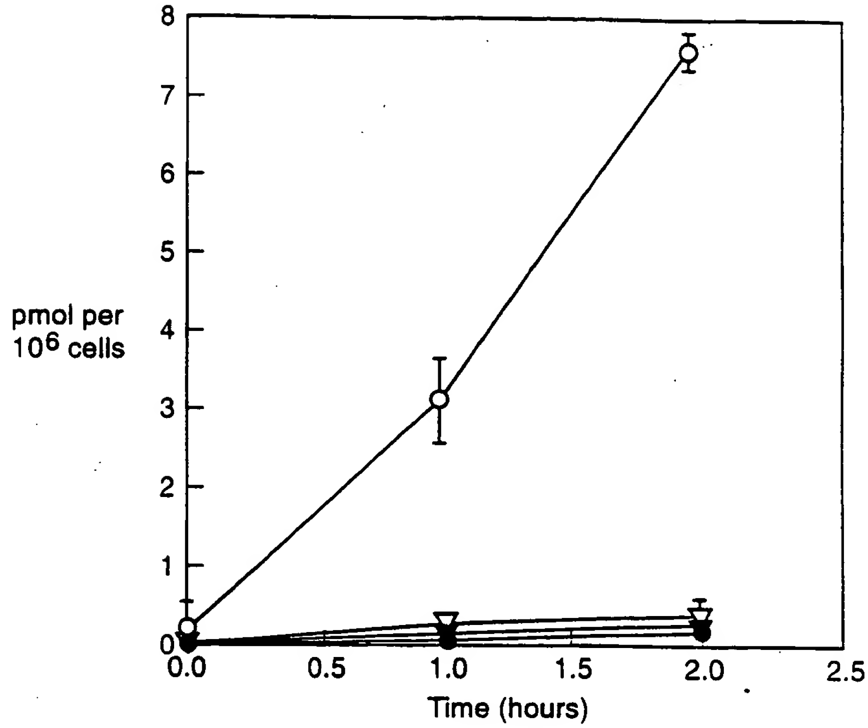

pmol per
10^6 cells
0
1
2
3
4
5
6
7
8
0.0
0.5
1.0
1.5
2.0
2.5
Time (hours)

FIG. 6

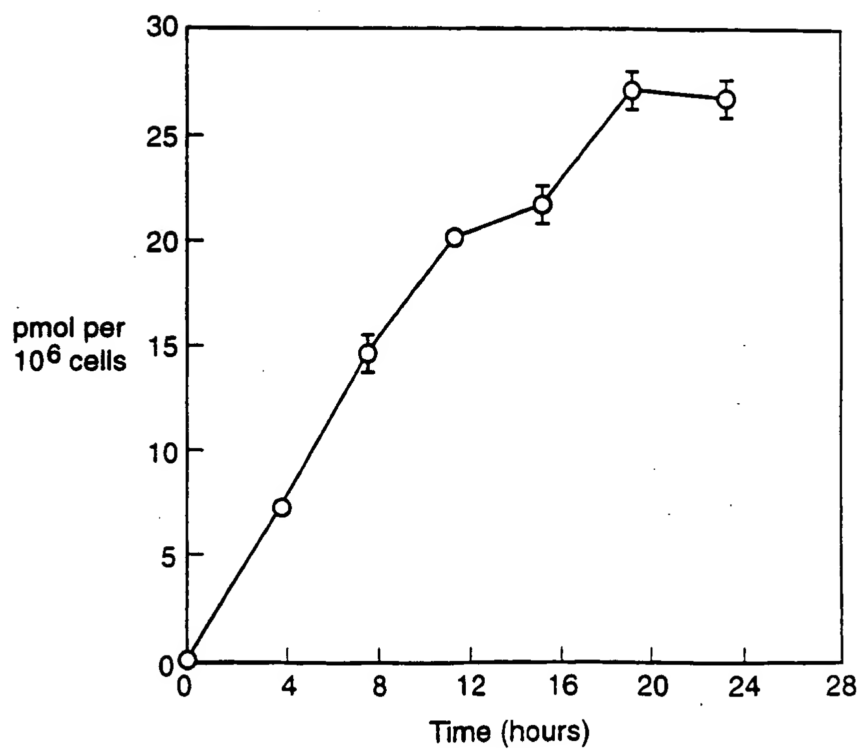

pmol per
10^6 cells
0
5
10
15
20
25
30
0
4
8
12
16
20
24
28
Time (hours)

FIG.7

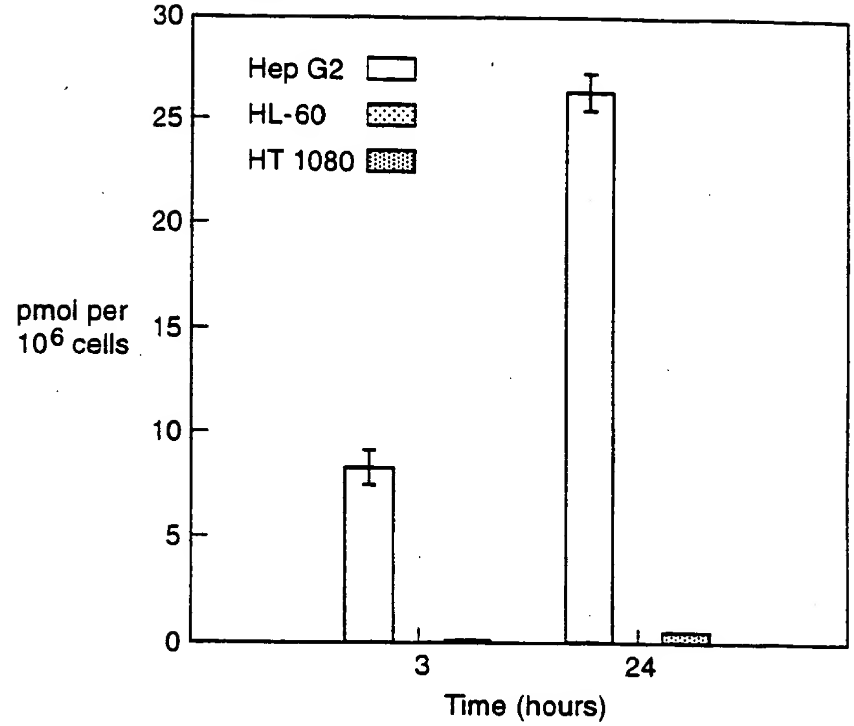

Hep G2
HL-60
HT 1080
pmol per 10^6 cells
30
25
20
15
10
5
0
3
24
Time (hours)

FIG. 8A

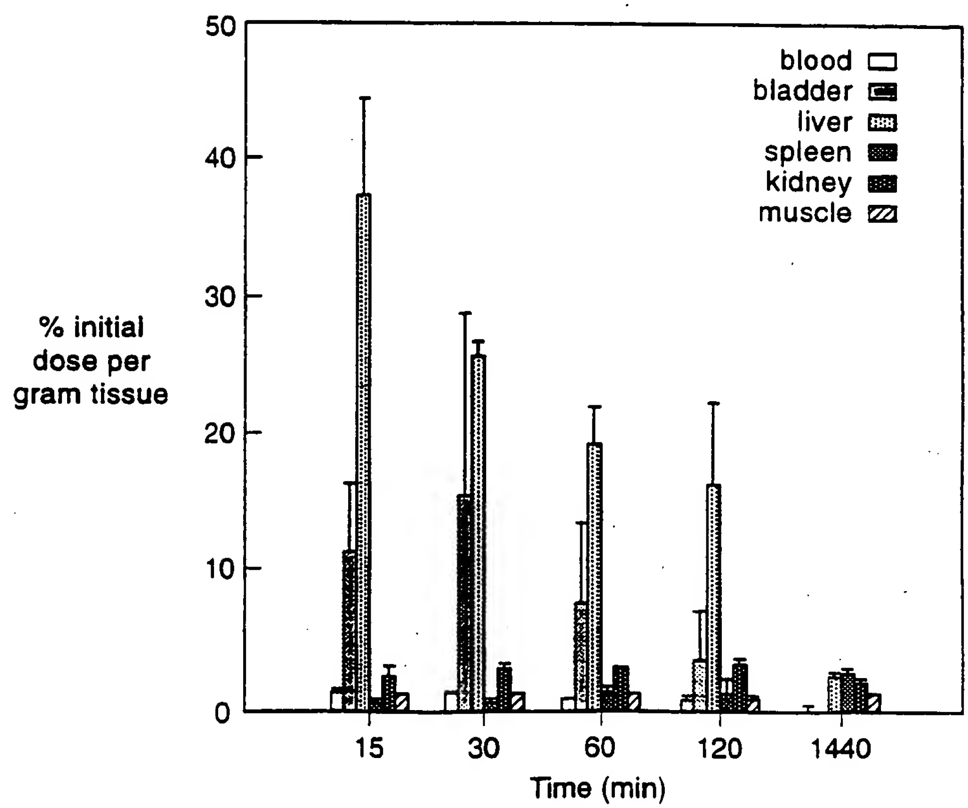

blood
bladder
liver
spleen
kidney
muscle
% initial dose per gram tissue
50
40
30
20
10
0
15
30
60
120
1440
Time (min)

FIG. 8B

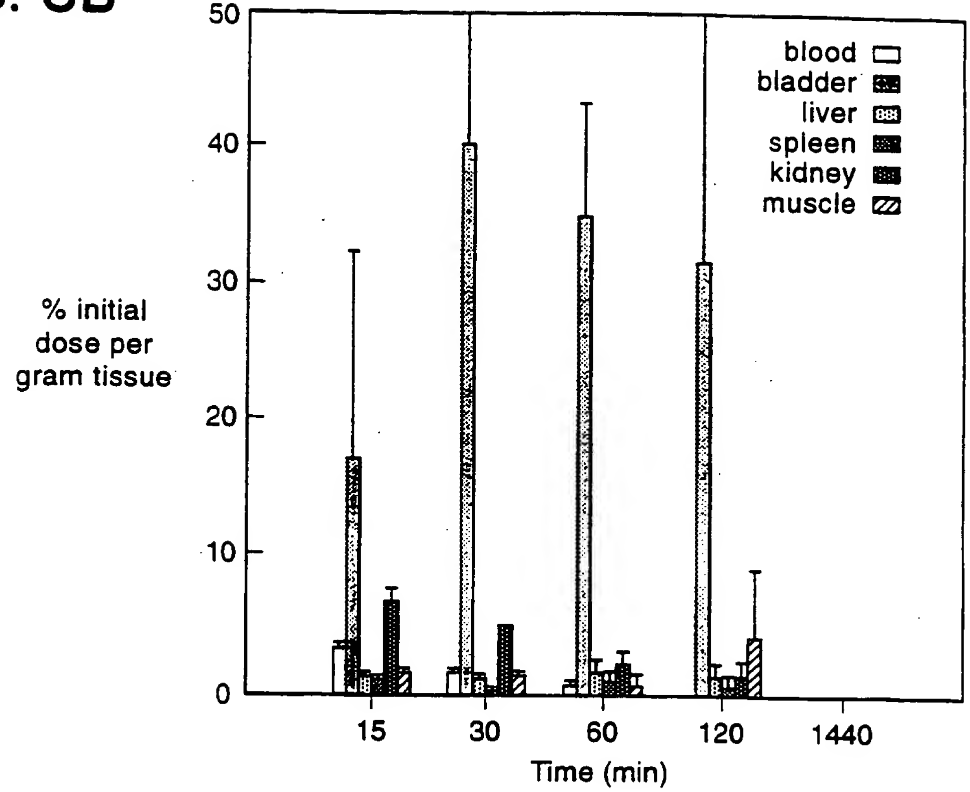

50
40
30
20
10
0
% initial dose per gram tissue
blood
bladder
liver
spleen
kidney
muscle
15
30
60
120
1440
Time (min)

FIG. 9

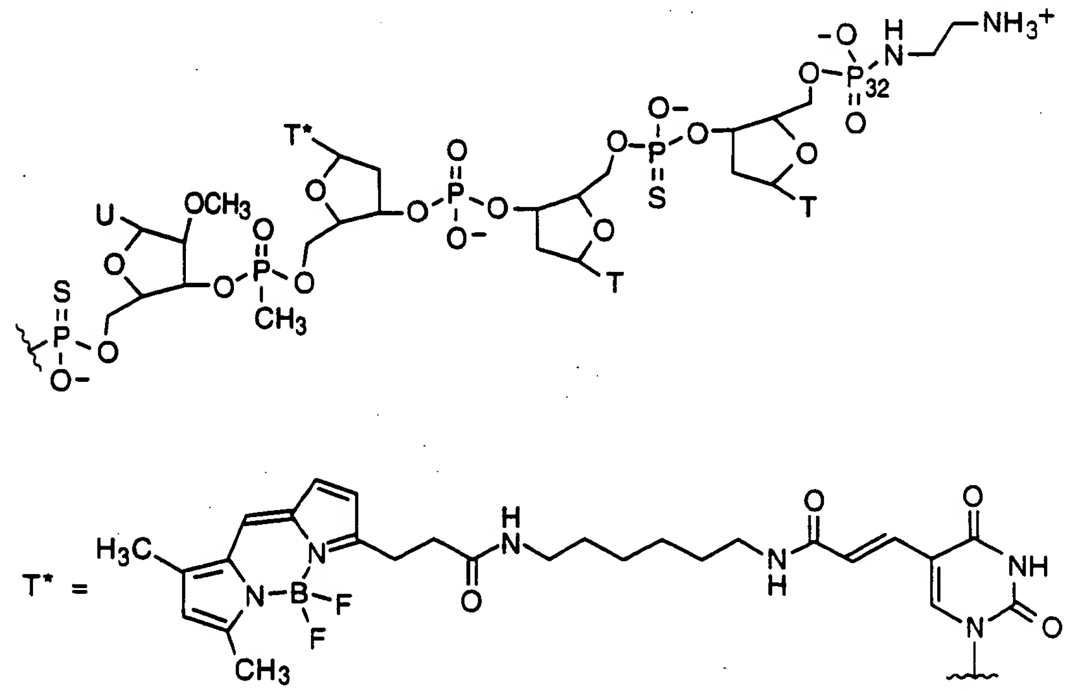

T* =

FIG. 10

$^{3'}$dT$^{5'}$-CPG

↓ Alternating couplings with 2'-OCH_3 methylphosphonate and 2'-OCH_3 phosphodiester synthons

$^{5'}$(2'-OMe-ApGpUpCpApGpUpCpApGpUpCpApGpU)dT* ps$^{3'-3'}$dTpsdT$^{5'}$-CPG

↓ C6-Disulfide cyanoethylphosphoamidite synthon

DMTO–$(CH_2)_6$–SS-$(CH_2)_6$-ps–$^{5'}$(2'-OMe-ApGpUpCpApGpUpCpApGpUpCpApGpU)dT* ps$^{3'-3'}$dTpsdT$^{5'}$-CPG

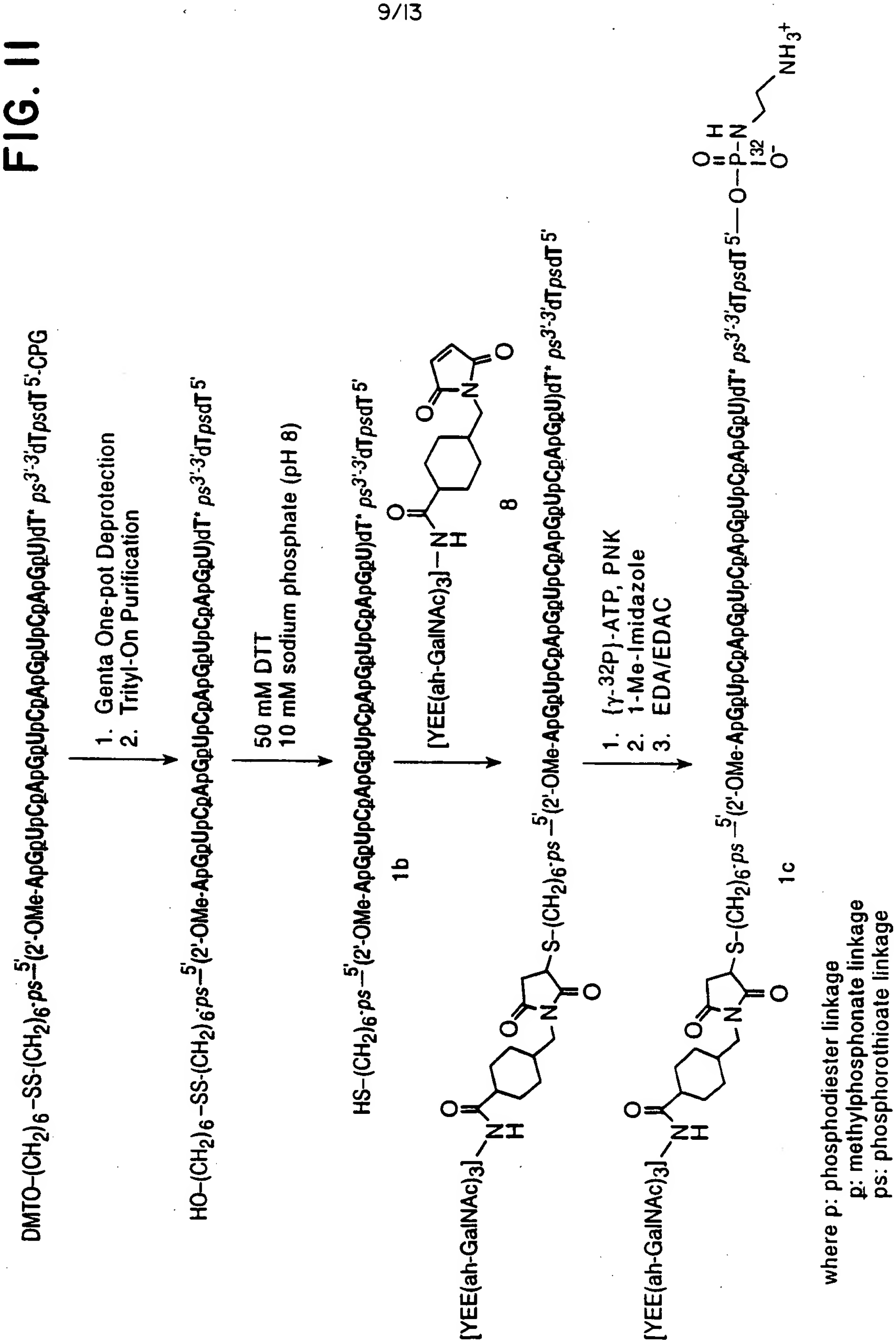

DMTO-(CH2)6-SS-(CH2)6-ps-5'(2'-OMe-ApGpUpCpApGpUpCpApGpU)dT* ps3'-3'dTpsdT5'-CPG
1. Genta One-pot Deprotection
2. Trityl-On Purification
HO-(CH2)6-SS-(CH2)6-ps-5'(2'-OMe-ApGpUpCpApGpUpCpApGpU)dT* ps3'-3'dTpsdT5'
50 mM DTT
10 mM sodium phosphate (pH 8)
HS-(CH2)6-ps-5'(2'-OMe-ApGpUpCpApGpUpCpApGpU)dT* ps3'-3'dTpsdT5'
1b
[YEE(ah-GalNAc)3]
8
S-(CH2)6-ps-5'(2'-OMe-ApGpUpCpApGpUpCpApGpU)dT* ps3'-3'dTpsdT5'
1. {γ-32P}-ATP, PNK
2. 1-Me-Imidazole
3. EDA/EDAC
NH3+
1c
where p: phosphodiester linkage
p: methylphosphonate linkage
ps: phosphorothioate linkage

FIG. 11

FIG. 12A

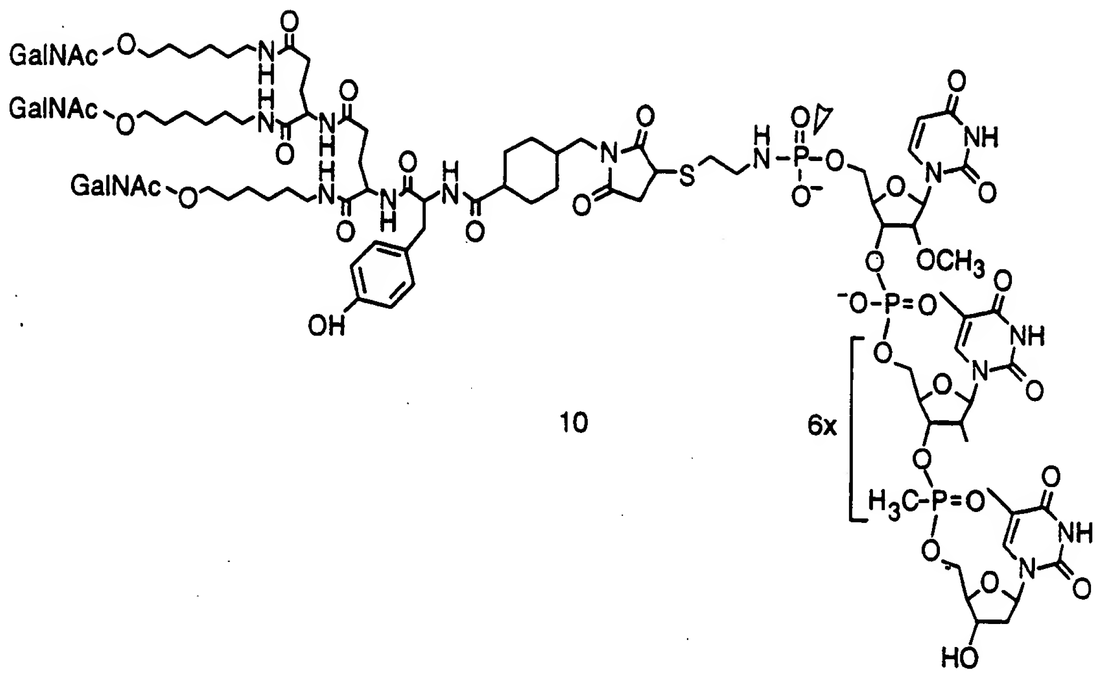


FIG. 12B

10: [YEE(ah-GalNAc)$_3$]-SMCC-AET-pU^{m}pI$_7$

12: [YEE(ah)$_3$]-SMCC-AET-pU^{m}pI$_7$

3: [Y]-SMCC-AET-pU^{m}pI$_7$

4: pU^{m}pI$_7$

5: [YEE(ah-GalNAc)$_2$]-SMCC-AET-pU^{m}pI$_7$

6: [YEE(ah-GalNAc)$_3$]-SMCC-AET-pU^m

FIG. 13

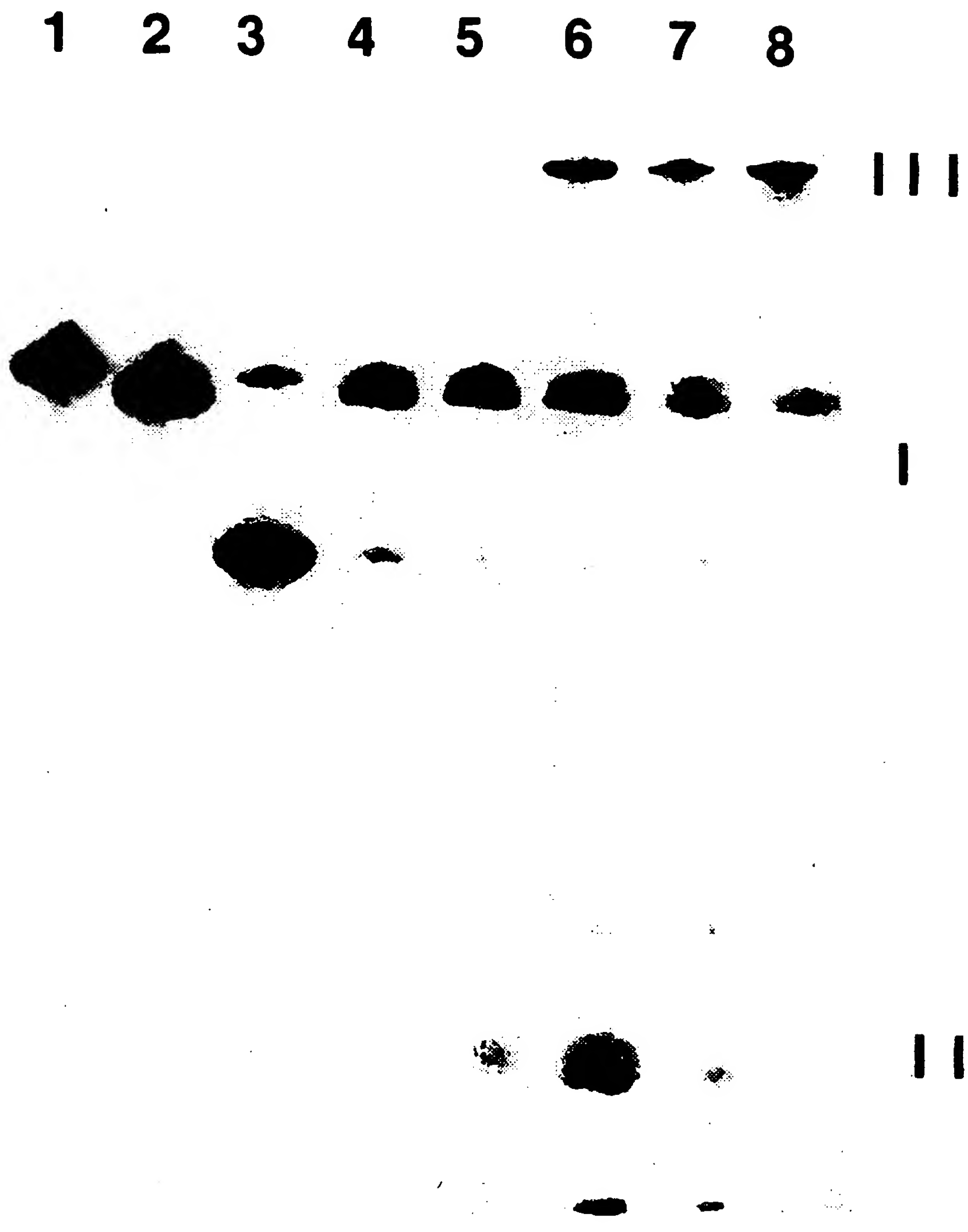
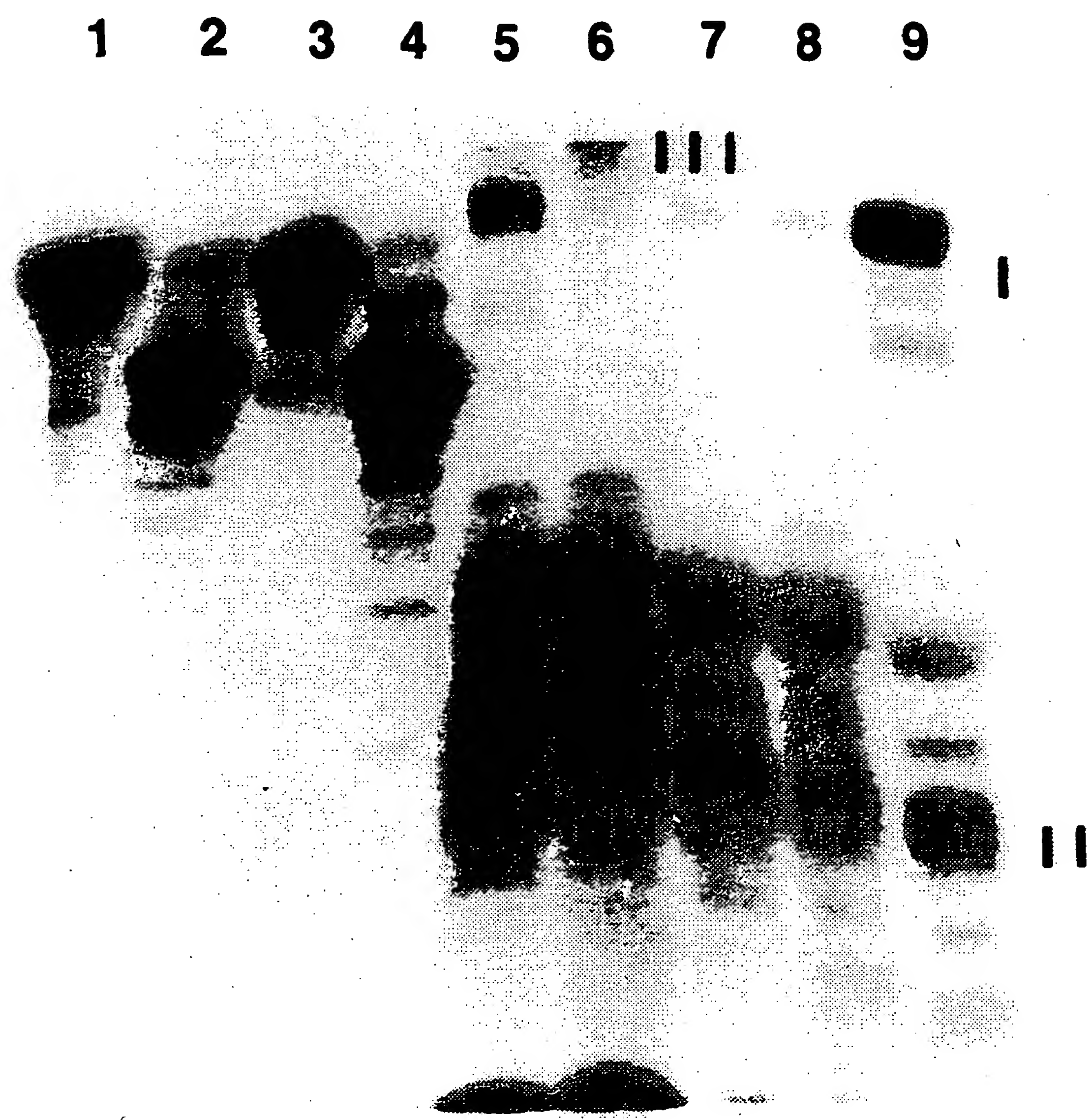


FIG. 14



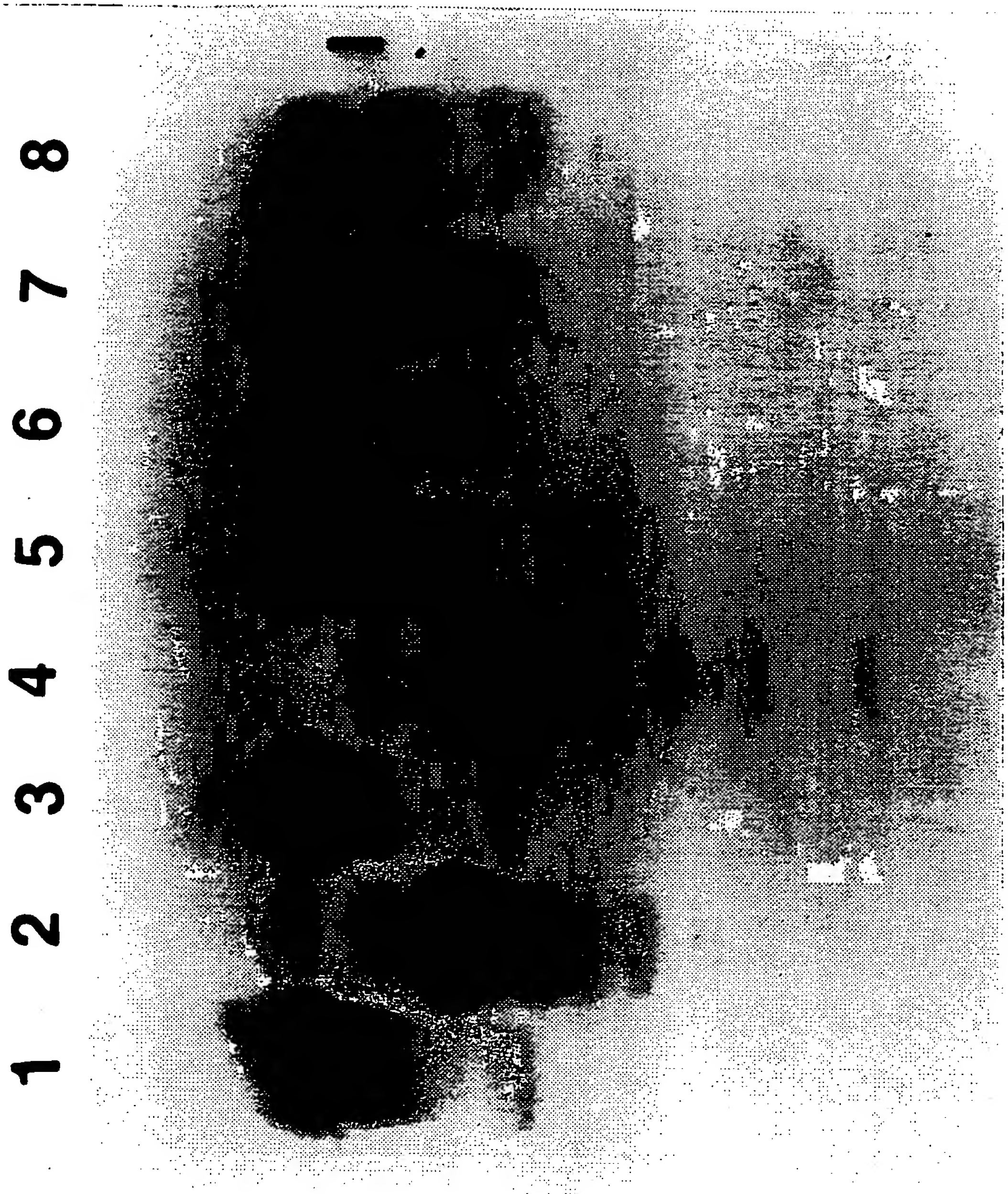


FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB96/01442

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70, 31/715, 31/735
US CL : 514/25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

aps, caplus, medlin, dialog search terms: drug targeting, neoglycopeptides, oligonucleosides, methylphosphonate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANGELAND, J. J. et al. Cell-type specific and ligand specific enhancement of cellular uptake of oligodeoxynucleoside methylphosphonates covalently linked with a neoglycopeptide, YEE(ah-GalNAc)$_3$. Bioconjugate Chem. 1995, Vol. 6, No. 6, pages 695-701, see entire document.	1-17
X --- Y	BONFILS, E. et al. Drug targeting: synthesis and endocytosis of oligonucleotide-neoglycoprotein conjugates. Nucleic Acids Research. 1992, Vol. 20, No. 17, pages 4621-4629, see entire document.	1-4 ---- 14
Y, P	US 5,554,386 A (GROMAN ET AL.) 10 September 1996, see entire document.	1-4, 11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search: 17 APRIL 1997

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